SUPPLEMENTARY MATERIAL

Protective Effect of *Epicatechin* on APAP-induced Acute Liver Injury of Mice through Anti-inflammation and Apoptosis Inhibition

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Abstract: *Epicatechin* (EC) is the most effective compound in *Euonymus alatus (Thunb.)Sieb*, and possesses a series of benefits, including anti-inflammatory, antioxidant, antiobesity and anticancer effects. In this study, we investigated the protective effects of EC in Acetaminophen(N-acetyl-p-aminophenol, APAP)-induced acute liver injury in C57BL/6J mice and explored the possible mechanisms involved in these effects.

Experimental

Reagents: EC was purchased from Yuanyebio (Shanghai, People's Republic of China LOT:C16N6Q5881). APAP and the positive drug, GSH were both purchased from Sigma-Aldrich (St Louis, MO, USA). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) microplate test kits were purchased from Jiancheng Bioengineering Institute (Nanjing, People's Republic of China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-6 were purchased from Becton Dickinson Co. (New Jersey, USA), and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies used in this study, including Bcl-2, Bax, Caspase-3 and β-actin were obtained from Cell Signaling Technology(Danvers, MA, USA).The First Strand cDNA Synthesis Kit was purchased from Thermo Scientific RewertAid (Waltham, MA，USA. All other chemicals used were either of analytical grade or of the highest purity commercially available.

Animals: Experiments were under the Chinese Guidance of Humane Use of Laboratory Animals. C57BL/6J mice(male, SPF，20±1g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, People's Republic of China).These mice were housed in an environment maintained at a temperature of 24°C±2°C and 55% humidity under a 12:12-hour light: dark cycle, with free access to a chow diet and water.

Drug administration: According to previous reports, polyethylene glycol was used to dissolve APAP and the resulting solution was administrated at a dose of 300 mg/kg by intraperitoneal injection to induce acute liver injury. The EC was dissolved in Carboxymethylcellulose sodium(CMC-Na, 0.5%) at the doses of 1,0.5 or 0.25 mg/kg and it was orally administered to mice twice daily for 2 days after the induction of acute liver injury(Yao et al.,2015; Liao et al.,2017).Sixty mice were randomly divided into six groups (n=10):

1) Control group: CMC-Na by lavage;
2) APAP group: APAP by intraperitoneal injection;
3) GSH group: GSH 1g/kg lavaged following with APAP by intraperitoneal injection;
4) Low EC group: EC 0.25 mg/kg lavaged following with APAP by intraperitoneal injection;
5) Middle EC group: EC 0.5 mg/kg lavaged following with APAP by intraperitoneal injection;
6) High EC group: EC 1 mg/kg lavaged following with APAP by intraperitoneal injection.

At the end of 2 days' administration, blood was collected from the eyeballs of mice. And the mice were euthanized by cervical dislocation, without the use of anesthetic. After that, the livers were exposed and excised, then kept at −80 °C until analyzed. The collected livers were used for histological assessment and the measurements of genes and proteins expression, respectively.

Liver index determination: Before being sacrificed, each mouse was weighed up. Similarly, the wet weight

of each liver was measured after sacrificed. The liver index=liver weight/ body weight×100%.

Biochemical analysis

Serum aminotransferase assay: After blood collection, the serum was separated by centrifugation at 4,200× g for 8 minutes at room temperature. An automated chemistry analyzer was used to access serum AST and ALT (MULTISKAN GO, Thermo).

Serum cytokine measurement: According to the instructions of each ELISA kits, the serum levels of TNF-α, IL-6 and IL-1β were measured by ELISA kits.

Histopathology: After soaking liver tissues in 4% paraformaldehyde for 24 hours, the tissues were embedded in paraffin. Cutting Sections (3 μm thick) for hematoxylin and eosin (H&E) staining, and observing the inflammation and tissue damage by a light microscopy.

Western blotting analysis: The liver tissues were removed from storage at -80°C and lysed with radioimmunoprecipitation assay lysis buffer and protease inhibitors. Bicinchoninic acid protein assay (Boster Biological Technology, Wuhan, People's Republic of China) was used to measure the protein concentrations. After that, the samples were boiled with 5× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer. According to standard protocols, the treated samples were separated by SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk (dissolved in TBS) for 1 hour and the blots were then incubated overnight at 4°C with the following primary antibodies: β-actin (1:1,000), Bcl-2 (1:1000), Bax (1:1000), Caspase-3 (1:1000).β-Actin was used as an internal reference for cytoplasmic proteins. All PVDF membranes were washed with TBST (TBS containing 0.1% Tween-20), then incubated with a secondary goat anti-mouse or anti-rabbit antibody (1:20,000) for 1 hour at 37°C. Finally, the membranes were washed with TBST three times for 15 minutes each time and detected the proteins with the Odyssey® two-color infrared laser imaging system (fluorescence detection).

Reverse transcription (RT)-PCR and quantitative real-time (qRT)–PCR: According to the instructions, 1 mL of TRIzol™ Reagent (Thermo Fisher Scientific) per 100 mg of liver tissues was added to the sample and homogenized by a homogenizer. 0.2 mL chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is precipitated from the aqueous layer with equal volume isopropanol. The precipitated total RNA is washed twice by 75% ethanol to remove impurities, and then resuspended by 0.3–0.6 mL DEPC-Treated Water for use in downstream applications. Then the reverse transcription kit (Thermo Fisher Scientific) was used to transcribe the RNA into cDNA. According to the SYBR Premix EX Taq instructions (TaKaRa Biotechnology), we used SYBR Green qRT-PCR with a 7900HT fast real-time PCR system (STRATAGENE, Made in Germany) to detect and analyze the relative

mRNA concentrations. The primers used in the PCR are listed in Table. β-actin was used as a reference control gene to normalize the expression value of each gene. Thermal cycler parameters were as follows: 1 cycle of 95 °C for 30 s, 40 cycles of denaturation (95 °C, 5 s) and combined annealing/extension (60 °C, 30 s). In the study, we used the method of '$2^{-\Delta\Delta Ct}$' to calculate the amount of the target gene. The formula is $2^{-\Delta\Delta Ct}=2^{-(\Delta Ct.q-\Delta Ct.cb)}$, $2^{-\Delta\Delta Ct}$ represents the amount of the target gene, the 'q' represents the sample, the 'cd' represents calibrator, and 'Δ' means to calculate the standard deviation.

Table Primer sequences used for polymerase chain reactions

Gene	Primer sequence (5′–3′)
TNF-α	
Forward	ACG GCA TGG ATC TCA AAG AC
Reverse	CGG ACT CCG CAA AGT CTA AG
IL-6	
Forward	CAT GTT CTC TGG GAA ATC GTG G
Reverse	GTA CTC CAG GTA GCT ATG GTA C
IL-1β	
Forward	CAT CCA GCT TCA AAT CTC GCA G
Reverse	CAC ACA CCA GCA GGT TAT CAT C
β-actin	
Forward	TAC CAC CAT GTA CCC AGG CA
Reverse	CTC AGG AGG AGC AAT GAT CTT GAT

TUNEL assay: TUNEL assay was used to detect the apoptosis of liver tissues. Paraffin-embedded sections (5 μm) were cut and deparaffinized, and the sections were digested with proteinase K (Sigma-Aldrich) at a concentration of 20 μg/mL for 15 minutes at room temperature. The slides were washed four times and then incubated with 2% hydrogen peroxide in PBS for 5 minutes at room temperature. After being washed twice, the slides were immersed in Terminal deoxynucleotidyl Transferase-containing buffer for 15 minutes. An antidigoxigenin antibody fragment carried a conjugated reporter enzyme (peroxidase) to the reaction sites, then localized peroxidase generated an intense signal from the chromogenic substrate diaminobenzidine. The counterstain was methyl green.

Statistical analysis: Data were expressed as mean ± standard deviation. ELISA and real-time PCR data were analyzed by one-way analysis of variance. The serum levels of ALT and AST, necrotic or edematous areas on histopathology, and Western blot were analyzed by Student's *t*-test. In all comparisons, $P < 0.05$ was considered statistically significant and $P < 0.01$ means very significant difference. All statistical analyses were calculated with GraphPad Prism version 6.0(GraphPad Software, Inc., San Diego, CA, USA).

Reference

Yao HT, Yang YC, Chang CH, et al.2015. Protective effects of (-)-epigallocatechin-3-gallate against acetaminophen-induced liver injury in rats. BioMedicine. 5:16-21.

Liao CC, Day YJ, Lee HC. 2017. ERK Signaling Pathway Plays a Key Role in Baicalin Protection Against Acetaminophen-Induced Liver Injury. The American Journal of Chinese Medicine. 45:1-7. DOI: 10.1142/S0192415X17500082.

Figure S1. EC rescued APAP-induced acute liver damage based on examination of ALT (A)and AST (B) levels. All data were expressed as Mean ± SD (n = 10). #P < 0.05 and ##P < 0.01compared with the control group; *P < 0.05 , **P < 0.01 and ***P < 0.001 compared with APAP-induced acute liver injury model group.

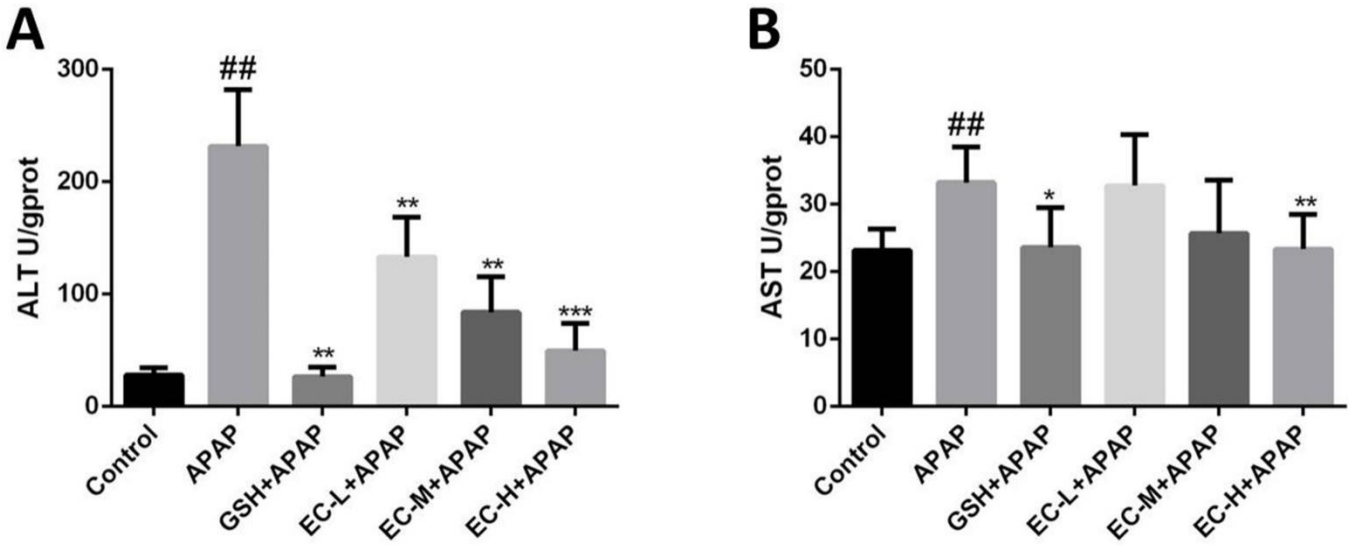
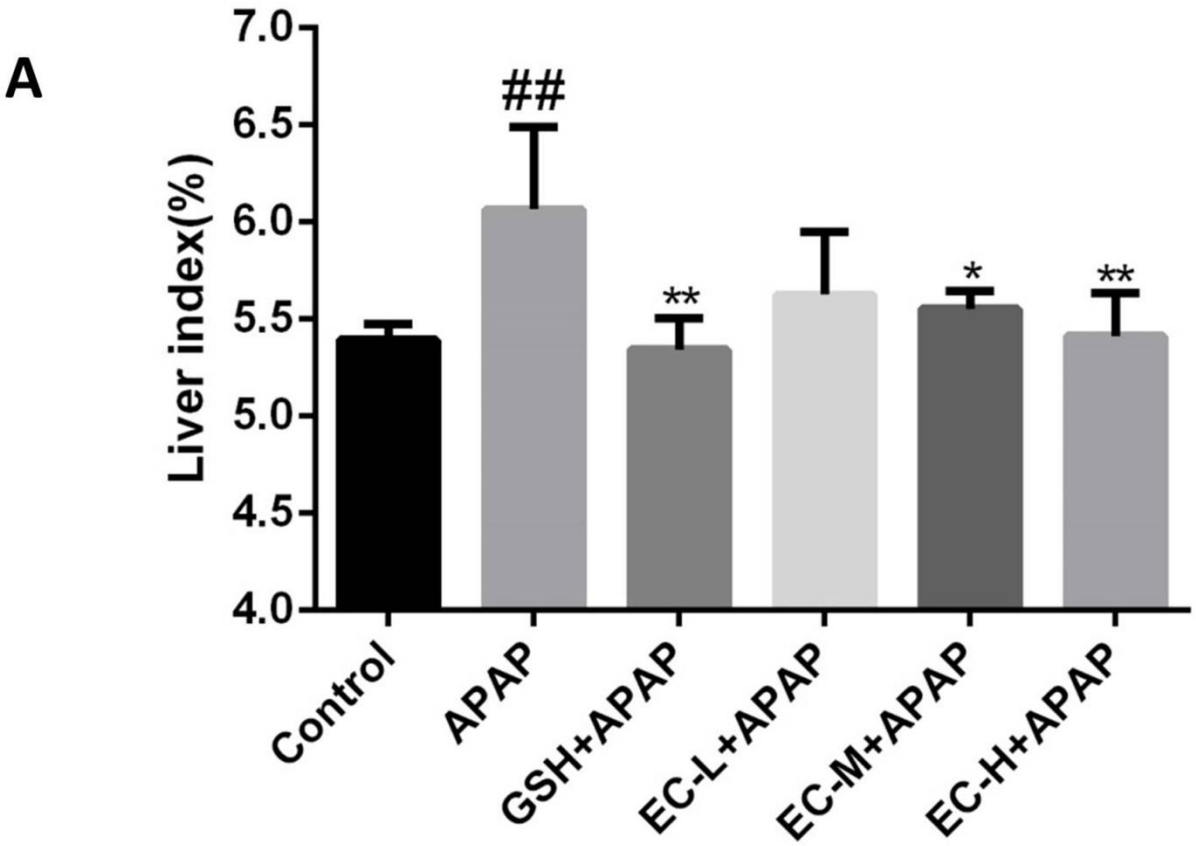


Figure S2.EC inhibited APAP-induced hepatomegaly and reduced liver index (A) as well as alleviated APAP-induced cell necrosis or cell death (B) through the evaluation by HE staining.



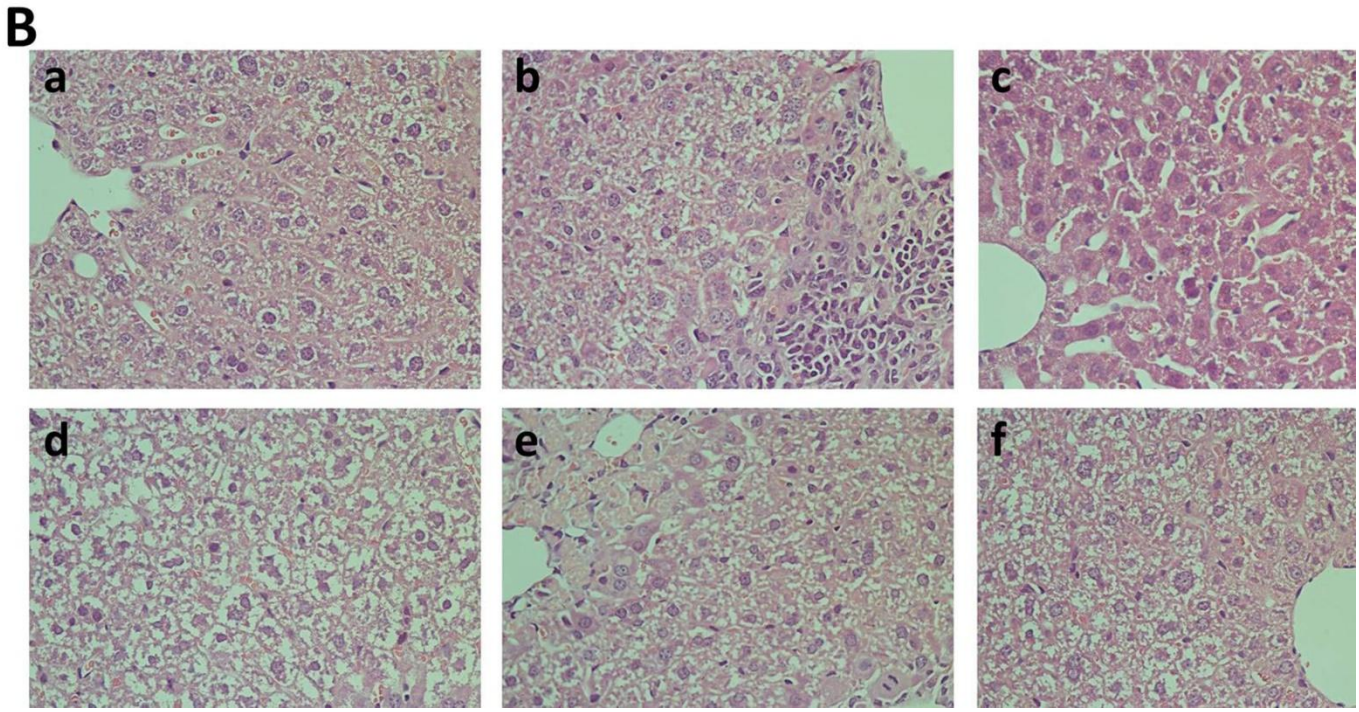


Figure S3. EC reduced inflammatory responses from APAP-induced acute liver injury in mice through the determination of mRNA expression of TNF-α, IL-6 and IL-1β by ELISA(A) and RT-PCR(B). All data were expressed as Mean ± SD (n = 3) from three independent experiments. #P < 0.05 , ##P < 0.01 and ###P < 0.001 compared with the control group; *P < 0.05 , **P < 0.01 and ***P < 0.001 compared with the APAP-model group.

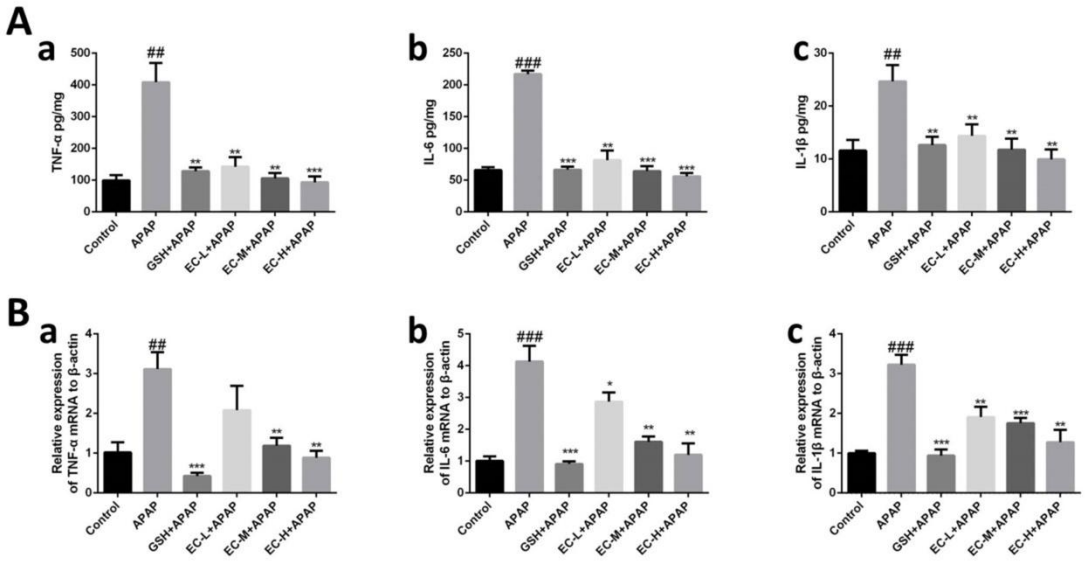


Figure S4. EC executed hepato protection through inhibited apoptosis. EC could result in down-regulation of Bax and Caspase-3 and up-regulation of Bcl-2 based on western blot analysis (A). Similarly, EC could obviously reduce liver cell apoptosis of mice induced by APAP based on the TUNEL staining (B) and its statistical analysis (C). ##P < 0.01 compared with the control group; *P < 0.05 and **P < 0.01 compared with the APAP-model group.